Functional dynamics of claudin expression in Japanese medaka (*Oryzias latipes*): response to environmental salinity

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Abstract

Salinity regulation of 13 claudin paralogs was investigated in osmoregulatory organs of euryhaline Japanese medaka. They were identified by blast-search in the medaka genome database based on representation in osmoregulatory organs of other teleosts. Our hypothesis was that, because of their sequence similarities to mammalian orthologs previously characterized as barrier- and ion-selective channel-forming proteins, these paralogs would respond to salinity according to expected modulation of osmoregulatory function. *Cldn10c*, *-10d*, *-10e*, *-10f*, *-27a*, *-28a*, *-28b* and *-30c* had 4- to 100-fold higher expression in gill than other examined organs. Two splice variants of *cldn10b* were predominantly expressed in kidney, while *cldn15a* and *-b* and *-25* were found mainly in intestine. In gills, *cldn27a*, *-28a*, *-28b* and *-30c* did not change between fresh water (FW) and seawater (SW)-acclimated fish, while *cldn10c*, *-10d*, *-10e*, *-10f* were most abundant in SW. Short-term SW transfer induced up-regulation of *cldn10* gill paralogs after 1 day, decrease in *cldn28b* and no difference for *cldn27a*, *-28a* and *-30c*. The reverse pattern was observed after FW transfer of SW medaka. Intestinal *cldn15a* and *-25* did not differ between FW and SW fish. However, *cldn15b* was 10-fold higher in FW than SW, suggesting a role in functional modulation of the intestine related to water and salt transport. In kidney, *cldn10bs* were elevated in SW fish, suggesting a role in paracellular ion transport in the marine nephron. Based on *in silico* analysis, most gill Cldn10s were predicted to form cation pores, whereas Cldn27a, 28a, 28b and 30c may increase epithelial resistance.

Key words: Euryhaline teleost, Gill, Intestine, Osmoregulation, Kidney, Paracellular ion transport, Tight junctions.

1. Introduction

Euryhaline fishes generally keep their internal osmotic concentration at about the same level (300-350 mOsm kg⁻¹) after acclimating to fresh water (FW: about 1-10 mOsm kg⁻¹) or seawater (SW; about 1050 mOsm kg⁻¹; Watts et al., 2001; Evans et al., 2005; Brauner et al., 2013) - a feat accomplished by the concerted action of osmoregulatory organs, mainly gill, intestine and kidney. Tight junction (TJ) strands make up the apico-lateral barrier, or gate, in between epithelial cells and regulate paracellular solute and water movement (Van Itallie and Anderson, 2006). In the gill, TJs between adjacent pavement cells (PVCs) and mitochondrion-rich cells (MRCs) are deep with multi-stranded connections (Sardet et al., 1979). In SW, shallow TJs connect neighboring MRCs and MRCs with adjacent accessory cells (ACs) suggesting a “leaky” paracellular pathway between these cells (Sardet et al., 1979). In the marine environment, branchial NaCl secretion involves transcellular chloride transport basolaterally by Na⁺,K⁺,2Cl⁻ cotransport and apically via cystic fibrosis transmembrane conductance regulator anion channel. This transport is secondarily coupled to the activity of Na⁺,K⁺-ATPase in the extensive tubular network of the MRCs (Karnaky et al., 1976). This anion transport is thought to generate a serosal positive transepithelial potential triggering sodium extrusion via a paracellular pathway (Evans et al., 2005; Edwards and Marshall, 2012). Passive uptake of water in the gut driven by active ion uptake compensates for osmotic water loss, while the kidney's main function in SW appears to be the excretion of excess divalent ions (Flik et al., 1996; Varsamos et al., 2004; Marshall and Grosell, 2006). In FW, fish are potentially subject to extensive ion loss and osmotic water gain. The gill epithelium has no MRC complexes or ACs and is generally considered as tight with a higher electrical resistance (Sardet et al., 1979; Wilson and Laurent, 2002). The branchial epithelium actively absorbs ions and the intestinal tract also allows for some ion uptake (mainly from food uptake; Marshall and Grosell, 2006). Meanwhile, the water load is countered by renal production of a large volume of hypotonic urine (Hickman and Trump, 1969).

TJs are composed of proteins like claudins (Cldns), occludin, junctional adhesion molecules and tricellulin. All have four trans-membrane spanning domains, two extracellular loops (ECLs), and a cytosolic carboxy-terminal linked to the cytoskeleton through adaptor molecules like those from the zonula occludens family (Gonzalez-Mariscal et al., 2008). Proteins belonging to the Cldn super-family determine the specific permeability and ion selectivity of TJs, as demonstrated

by over-expression of exogenous Cldns in epithelial cell cultures (Furuse et al., 1998; Van Itallie et al., 2001). Furthermore, site-directed mutageneses have shown that the specific paracellular ion movement is influenced by charged amino acids (aa) of the first ECL (ECL1) and intercellular homo- or heterotypic Cldn-Cldn interaction (Colegio et al., 2003; Angelow et al., 2008). In this way, they form permeability-selective barriers critical to the function of a specific tissue. While 27 *cldn* genes have been described in mammals (Günzel and Yu, 2013), genomic and tandem gene duplication events have contributed to an expanded Cldn family in teleost including 54-56 different genes (Loh et al., 2004; Baltzegar et al., 2013). Cldns are often developmentally regulated and expressed in a tissue- and cell-specific manner (Loh et al., 2004; Tipsmark et al., 2008b).

In teleost gills, SW-acclimation induces elevated expression in *cldn10d* and *cldn10e* (Tipsmark et al., 2008b; Bui et al., 2010). These paralogs are interesting candidates potentially involved in formation of a cation-selective pathway associated with Na⁺ extrusion in SW, especially since the homolog in mouse was shown to form cation-selective pores (Cldn-10; Günzel et al., 2009). Mouse Cldn4 appears to share a common ancestor with 13 teleost Cldns (Baltzegar et al., 2013) of which a number are highly expressed in the gill (*cldn27a*, *cldn28a-b*, *cldn30c*) in FW or in both FW and SW (Tipsmark et al., 2008a, 2008b). Of these, Cldn30c has been shown to be involved in tightening of fish epithelia (Engelund et al., 2012), similar to the barrier-forming mammalian homologs (Van Itallie et al., 2001; Yu et al., 2003).

The Japanese medaka (*Oryzias latipes*) is a euryhaline teleost of the ricefish family. This fish species generally lives in FW (Sakamoto et al., 2001), but is able to acclimate to full strength seawater (Inoue and Takei, 2002, 2003; Kang et al., 2008). Its euryhaline capacity combined with an evolutionary history diverging from the well-studied stenohaline zebrafish (*Danio rerio*), makes this species a particularly good model to study osmoregulation. Japanese medaka has the advantage of being easy to rear. Furthermore, its genome is fully sequenced and annotated, and relatively small compared to other teleosts (Tanaka, 1995). Medaka easily acclimates to salinity challenges in a manner similar to other euryhaline teleost and a recent study revealed the existence of three types of branchial ionocytes in FW and one in SW (Hsu et al., 2014). However to date, no information is available on the functional expression of TJ proteins in this species.

The objectives of the present study were first to identify *Cldn* paralogs involved in regulation of paracellular permeability in osmoregulatory organs of Japanese medaka, with special focus on the gill. Our driving hypothesis was that fish paralogs sharing sequence similarity in the ECL1 of mammalian orthologs with barrier or ion-selective properties would respond to salinity according to the expected changes in osmoregulatory function. Based on their representation in the gill, kidney and intestine of other teleosts, 13 paralogs were pinpointed for this study. The chosen paralogs' nomenclature was examined using phylogenetic analysis and their functional properties predicted based on *in silico* analysis. This was followed by determination of organ distribution and expression patterns in osmoregulatory organs of fully FW- or SW-acclimated fish. Expression of *cldns* with high gill levels was examined during the initial stages of salinity acclimation. This was done in two transfer experiments (from FW to SW, or SW to FW) with sampling after 1 and 3 days.

2. Materials and Methods

2.1 Animals

Adult Japanese medaka (*O. latipes*, Temminck & Schlegel; size range: 25-35 mm, 250-350 mg) were obtained from the Aquatic Research Organisms (Hampton, NH, USA). They were maintained in 150 L tanks filled with aerated de-chlorinated tap water, mechanically and biologically filtered (0.34 mM Na⁺, 0.64 mM Ca²⁺, 0.09 mM Mg²⁺, 0.03 mM K⁺). They were kept under a 14 hrs light/10 hrs dark photoperiod, at 20°C. Fish were fed 3 times per day with Tetramin Tropical flakes (Tetra, United Pet Group, Blackburg, VA, USA) or frozen brine shrimp (San Francisco Bay Brand, Inc., Newark, CA, USA). Food was withheld during the short-term salinity transfer experiments from one day before and throughout the experiment. All experiments and handling procedures were approved by the Animal Care and use Committee of the University of Arkansas (IACUC protocol number 11005).

2.2 Experiments and sampling

Three experiments were performed in order to examine the chosen *cldns* organ distribution and response to salinity. One experiment applied long-term FW- and SW-acclimated (28 ppt) fish to determine the organs levels of medaka *cldns* at the two conditions. Fish were acclimated for one

month at their respective salinity prior to sampling. They were anesthetized using 100 mg L⁻¹ of tricaine methanesulfonate (MS-222, buffered with NaHCO₃ for FW) and then killed by cervical dislocation. Gills, kidney, intestine, muscle and liver were dissected, immediately frozen on dry-ice and stored at -80°C before further use. Samples of each organ from FW were used in order to determine the distribution of claudins (*N* = 6). Samples from the osmoregulatory organs (gills, kidney and intestine) were used to determine *cldn* expression according to salinity (*N* = 6). The initial gill response to salinity was examined in two short-term transfer experiments: fishes long-term acclimated for at least one month were transferred from FW to SW or SW to FW and sampled after 1 and 3 days. As sham controls, fish were transferred from FW to FW or SW to SW.

2.3 Claudin nucleotide sequences, alignment and phylogenetic analysis

The claudin targets mRNA sequences in medaka were found and identified by similarity search BLAST against known claudins of tiger pufferfish, *Takifugu rubripes* (*Fugu*), using the database GenBank (Table 1). The phylogenetic tree construction was based on maximum-likelihood analysis of putative aa sequences of medaka (Me), *T. rubripes* (Fu) and mammalian (house mouse, *Mus musculus*, Mo), and as out-group the clarin1 from mouse (Table 2). The maximum likelihood consensus tree was generated using SEQBOOT, PROML and CONSENSE, all programs in the PHYLIP package (Felsenstein, 1989). A neighbor joining consensus tree was generated using SEQBOOT, PROTDIST, NEIGHBOR and CONSENSE also programs in the PHYLIP package. Protein sequences were aligned using ClustalW2 pairwise alignment algorithm.

2.4 RNA isolation, cDNA synthesis and quantitative PCR (qPCR)

Organs were homogenized in Tri reagent (Sigma-Aldrich, St. Louis, MO, USA) using a VWR PowerMax 200 rotating knife homogenizer (Advanced Homogenizing System, Manufactured by pro Scientific for Henry Troemner LLC, Thorofare, NJ, USA). Total RNA was extracted according to the manufacturer's protocol. RNA pellet was dissolved into nuclease-free water and the concentration and purity (A260/A280 ratio at least > 1.90) were estimated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). For each sample, first strand cDNA was obtained from 1 µg of total RNA using the High Capacity cDNA reverse

transcription kit from Applied Biosystems (Foster City, CA, USA) in a final volume of 20 µL and following the manufacturer's protocol. Claudins mRNA sequences used for the design of specific primers were: *Me-cldn10b1*, *Me-cldn10b2*, *Me-cldn10c*, *Me-cldn10d*, *Me-cldn10e*, *Me-cldn10f*, *Me-cldn15a*, *Me-cldn15b*, *Me-cldn25*, *Me-cldn27a*, *Me-cldn28a*, *Me-cldn28b* and *Me-cldn30c* (Table 1). All primers used in this study, including those used as reference genes for normalization (ribosomal protein P0, *rplp0*; elongation factor 1α, *ef1a*; β-actin, *actb*), were designed using Primer-3 software (Rozen and Skaletsky, 1998) and synthesized by Sigma Aldrich. Quantitative real-time PCR (qPCR) analyses were performed using BioRad CFX96 platform thermocycler (BioRad, Hercules, CA, USA), the SYBR® Green JumpStar™ Taq ReadyMix™ (Sigma-Aldrich) and a primer concentration of 150 nM each, in a final volume of 15 µL. The qPCR was performed with an initial heating step at 94°C for 2 min, followed by 40 cycles of: denaturation step at 94°C for 30 sec, and an annealing/elongation step at 60°C for 1 min. Melting curve analysis was performed from 55 to 94°C with a gradient of 5 s per °C to confirm the specificity of the reaction. Standard curves were used to determine the primer sets efficiency. Relative mRNA expression was calculated according to Pfaffl's equation (Pfaffl, 2001): $C_n = (1 + E_a)^{-C_t}$, with C_n the relative copy number, C_t the threshold cycle of the target gene and E_a the amplification efficiency. Relative expression of target genes was normalized against the geometric means of the corrected expression data of the three normalization genes using GeNorm software (Biogazelle, Zwijnaarde, Belgium). No template controls (NTC) were always included to confirm specificity of template amplification and absence of primer-dimers.

2.5 Statistics

All statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA, USA). The homogeneity of variances was tested with Bartlett's test. To meet the ANOVA assumption of homogeneity of variances, transformation of data was done when required. Organ expressions were analyzed by one-way ANOVA, followed by Tukey's Honestly Significant Difference (HSD) post-hoc test. Expression data from the short-term experiments were analyzed with two-factorial ANOVA. When a significant interaction between factors was found, this was followed by Bonferroni-adjusted Fisher's LSD pairwise comparison test and otherwise the overall effects are indicated. Results are expressed as the mean ± standard error mean (s.e.m.) with a p-value of $p < 0.05$.

3. Results

3.1 Phylogenetic analysis

Thirteen Japanese medaka Cldns were classified according to their similarity with orthologous genes in *Fugu*. Six genes, including 2 splice variants, encoded Cldn10 proteins (*Me-cldn10b1*, *-10b2*, *-10c*, *-10d*, *-10e* and *-10f*). Me-cldn10 paralogs are highly conserved in teleosts and exhibit 74-97% of amino acids (aa) sequence identity compared to *Fugu* Cldn10's (data not shown). The nomenclature of the medaka claudins was examined by constructing a maximum likelihood phylogenetic tree based on the coding sequences of *Fugu* class II and IV Cldns (classification by Loh et al. 2004; Fig. 1) and mouse Cldns. The consensus phylogenetic tree confirmed the initial chosen nomenclature (Fig. 1) and a neighbor-joining analysis gave a similar result (data not shown). One Cldn10 paralog was most closely related to *Fugu* Cldn10e but still distinctively different and thus given the name Cldn10f (see Fig. 1). The tree showed that Me-Cldn10s are grouped with *Fugu* Cldn10 homologues and mouse Cldn10 (a and b splice variant). Medaka Cldn15a and b are grouped with their respective *Fugu* Cldn15a and b, along with mouse Cldn15. Medaka Claudin27a, -28a, -28b and -30c are grouped with their respective *Fugu* homologue and mouse Cldn3 and -4.

3.2 In silico prediction of claudin functional properties

The first ECL of each claudin was aligned with mammalian sequences, for which functional investigations have been undertaken in previous studies, in order to deduce putative functional role of these proteins in medaka (Fig. 2). The ECL1 consists of about 50 aa and contains positively (blue; +) and negatively (red; -) charged residues, as well as aromatic residues (aaa). Some of these amino acids are highly conserved, such as the first tryptophan residue of our alignment, which was numbered 1 for clarity. The bracket indicates the area after the 2nd conserved Cys residues especially involved in selective permeability. Residues highlighted in yellow indicate positions essential for ion selectivity. Aromatic residues are conserved in all the claudins studied at the position 1, 21, 25 and 41, with the exception of Leu21 and Ile41 for Me-Cldn10b2 and 10c, respectively. Me-Cldn10b1, -10d, -10e, and -10f share the same suite of residues adjacent to the second conserved Cys residue of the ECL1 (bracket area from 39-42, Fig. 2) as Mo-Cldn10b: +/-/aaa. This pattern is also found in Me-Cldn15a, -15b and -25. Me-

Cldn10b2 however has a positive residue followed by two aaa at the position 39-42, which is similar to Mo-Cldn10a, while Me-Cldn10c only has a positive residue in this area. Furthermore, Me-Cldn10b2 shared an Arg (position 6) with Mo-Cldn10a. Finally, Cldns of mouse and teleosts of class VI shared a similar motif at 39-42 positions: +/-neutral (or aaa for Me-Cldn28b)/aaa/-.

3.3 Organ distribution

The transcript abundance of thirteen claudins was analyzed and compared in gills, kidney and intestine as well as in liver and muscle (Fig. 3). The mRNA level of each claudin was generally distinctly higher in one particular osmoregulatory organ. The highest expression of *Me-cldn10c*, *-10d*, *-10e*, *-10f*, *-27a*, *-28a*, *-28b* and *-30c* was found in gills (Fig. 3C-F, J-M) with levels 4 to 100-fold higher than in kidney or intestine. *Me-cldn10b1* (about 14-fold the gill level) and *10b2* (about 17-fold the gill level) were mainly expressed in kidney (Fig. 3A, B). Meanwhile, *Me-cldn15a*, *-15b* and *-25* had 16 to 2700-fold higher expression in intestine than in the other examined organs (Fig. 3G-I). Most of the examined claudins had a very low expression in liver and muscle (*Me-cldn10b1*, *-10b2*, *-10f*, *-15a*, *-15b*, *-25*, *-27a*, *-28a*, *-28b* and *-30c*) and *Me-cldn10c*, *-10d* and *-10e* are undetectable or barely expressed in organs other than gills.

3.4 Claudin expression in FW and SW

Each claudin was quantified in the osmoregulatory organs of fish acclimated long-term to FW vs. SW (Fig. 4). *Me-cldn10c*, *-10d*, *-10e* and *-10f*, which are found predominantly in gills, showed a significant 3-4-fold higher mRNA expression in SW than in FW (Fig. 4C-F). However, the four other claudins highly expressed in gills (*Me-cldn27a*, *-28a*, *-28b*, *-30c*) did not differ between FW and SW (Fig. 4J-M). *Me-cldn15a* and *-25* which are mainly expressed in the intestine had similar expression in FW and SW, while *Me-cldn15b* was about 8-fold higher in FW than in SW fish (Fig. 4G-I). In the kidney, both *Me-cldn10b* splice variants (1 and 2) had elevated expression in SW fish being 3-10-fold higher than in FW (Fig. 4A, B).

Transcript expression of the claudins with dominant gill expression (*Me-cldn10c*, *-10d*, *-10e*, *-10f*, *-27a*, *-28a*, *-28b* and *-30c*) was quantified in the initial acclimation stages after FW to SW transfer (Fig. 5) or SW to FW transfer (Fig. 6). SW transfer induced an overall elevation of *Me-cldn10c*, *-10d*, *-10e* and *-10f* expression (Fig. 5A-D). The lack of interaction between time and

salinity signify that the observed salinity effects are similar at 1 and 3 days. There were no significant effects on *Me-cldn27a*, *-28a* and *-30c* (Fig. 5E, F, H). There was an overall effect of salinity on *Me-cldn28b* with reduced levels in the SW-transferred fish (Fig. 5G). After FW transfer of SW-acclimated medaka, an overall effect of salinity with reduced levels of *Me-cldn10c*, *-10e* and *-10f* expression was found. The lack of interaction between time and salinity shows that the observed salinity effects were similar after 1 and 3 days (Fig. 6A, C, D). For *Me-cldn10c*, there was a significant interaction between time and salinity, and reduced mRNA levels were first observed after 3 days in FW (Fig. 6B). There were no significant effects of FW transfer on *Me-cldn27a*, *-28a* and *-30c* (Fig. 6E, F, H). However, *Me-cldn28b* was elevated after 1 and 3 days (overall effect of FW).

4. Discussion

4.1 Overview

Recently, Japanese medaka has been highlighted as a good alternative model to zebrafish for osmoregulation studies and multiple proteins involved in transcellular solute and water transport have therefore been the topic of a number of studies (Kang et al., 2008; Wu et al., 2010; Lin et al., 2012; Madsen et al., 2014; Hsu et al., 2014). The expression of the TJ proteins defines the paracellular barrier and thus their involvement in overall epithelial function has yet to be examined. In the present work, 13 Cldn sequences were identified in the genome of the Japanese medaka with predicted role in osmoregulation (Chasiotis et al., 2012). The Cldns were named based on phylogenetic analyses and following the nomenclature used in other teleost species (Bagherie-Lachidan et al., 2008, 2009; Tipsmark et al., 2008a, 2008b), as outlined in *Fugu* (Loh et al., 2004) and Zebrafish (Baltzegar et al., 2013). Eight genes encoding for Me-Cldn10s (i.e. *Me-cldn10c*, *-10d*, *-10e* and *-10f*) and *Me-Cldn27a*, *-28a*, *-28b* and *-30c* had the highest mRNA expression in gills. *Me-cldn10b* had a high level of renal expression while *Me-cldn-15a*, *-15b* and *-25* were all strongly expressed in the intestine. A summary of our results and the putative role of each medaka claudin are presented in Table 3. The salinity-sensitive expression of some *Me-cldns* suggests an important role in the phenotypic plasticity of the gill, kidney and intestine.

4.2 Putative function of Claudin paralogs in medaka

The phylogenetic analysis and ECL1 alignment with known *Fugu* and mammalian claudins give us some insight into the potential function of the medaka Cldns. Indeed, the ECL1 creates a barrier or anion/cation pore according to its composition of charged and aromatic amino acids (see yellow highlighted residues, Fig. 2; Colegio et al., 2002; Van Itallie et al., 2003; Angelow et al., 2008; Li et al., 2013). As such, when Mo-Cldn15 D31 or/and E40 are substituted by a positive charge, the mutated protein displays a high anion permeability creating chloride-selective paracellular channels (Colegio et al., 2002; Angelow et al., 2008). Furthermore, Li et al. (2013) demonstrated that the aromatic residue facilitates cation permeation by side chain cation- π interaction and limits anion movement by restricting the pore size for cation-selective claudins. These studies and our alignment analysis substantiate the hypothesis that four Claudin-10s in Japanese medaka (i.e. Me-Cldn10b1, -10d, -10e and -10f) and two Cldn15s are likely to be cation pore-forming proteins. Me-Cldn10b2 possesses a very similar ECL1 to Mo-Cldn10a with the same aa (position 6 and 39) which have been shown to be the only positive charges influencing chloride permeation in mammals (Van Itallie et al., 2006). Furthermore, Li et al. (2013) suggested that aa's located after the 2nd conserved Cys in anion-selective claudins facilitate Cl⁻ movement and create a strong steric effect, preventing Na⁺ passage. These similarities strongly suggest that Me-Cldn10b2 could form an anion-selective pore. The function of Cldn10c and -25 in medaka is more uncertain due to mutations, in particular at position 31 and 40, respectively. However, Colegio et al. (2002) showed that D31 is sufficient to confer to the protein the cation-selective properties while D40 alone increases sodium permeability; together they have an additive effect. Claudins of class VI are orthologous to the mammalian Claudin-4 previously described as a cation barrier. Since all Me-Cldn4-like paralogs possess the same motif of aa's from position 39 to 42 which is responsible, at least in part, for the cation resistance (Colegio et al., 2002), we hypothesize that Me-Cldn27a, -28a, -28b and -30c form cation barriers. This is in agreement with their high similarity to a barrier forming paralog from Atlantic salmon and zebrafish (Engelund et al., 2012; Kwong and Perry, 2013).

4.3 Claudins in the medaka gill

Four *cldn10* paralogs (*c*, *d*, *e* and *f*) found in medaka are mainly expressed in the gills. Except for *cldn10f*, these TJ proteins have been detected in the gills of several euryhaline species such as Atlantic salmon (Tipsmark et al., 2008a; Tipsmark et al., 2009), rainbow trout (Kolosov et al.,

2014), *Fugu* (Loh et al., 2004) and spotted green pufferfish (Bui et al., 2010). All four claudins were most abundantly expressed in gill of SW medaka with a response to salinity change within 1 day of exposure. Seawater also caused an elevation of Cldn10d and -10e abundance in spotted green pufferfish (Bui and Kelly, 2014) and of *cldn10e* levels in Atlantic salmon (Tipsmark et al., 2008b). In rainbow trout, *cldn10c* and -10d are detected only in cultured epithelium containing both PVCs and MRCs but not in PVCs-only epithelium (Kolosov et al., 2014), suggesting that these paralogs are exclusively found in association with ionocytes. Similarly, the elevated expression in SW of medaka *cldn10c*, *10d*, *10e* and *10f* paralogs suggest a specific role associated with cell types that proliferate in this environment. Thus, we speculate that they are essential to the functioning of SW-type MRCs. As discussed above, *in silico* predictions suggest that they all have the potential to form intercellular cation-selective pores. Thus, one possible role of one or more of these Cldn10 paralogs could involve sodium exit through the TJs linking ACs and MRCs.

The medaka homologues (*cldn27a*, -28a, -28b and -30c) to mammalian Cldn4 are all highly expressed in gills, as previously found in Atlantic salmon (Tipsmark et al., 2008b) and is also seen in several other teleosts (Chasiotis et al., 2012). In some species, *cldn27a*, -28a, -28b and -30c are regulated by salinity and hormone treatments (Chasiotis et al., 2012). In Atlantic salmon, SW acclimation induces a decrease in transcript levels of *cldn27a* (Tipsmark et al., 2008b) and it has been suggested to be a tightening TJ protein. In contrary, gill *cldn27a* increased in spotted green pufferfish after acclimation to SW (Bagherie-Lachidan et al., 2009). In medaka, no effect of salinity on branchial *cldn27a* expression has been seen during both initial and long-term acclimation. In Atlantic salmon and tilapia, SW-acclimation induced a decrease in *cldn30c*. FW-acclimation induced an up-regulation of *cldn28b* in tilapia, while no overall effect on *cldn28a* and -28b were detected in Atlantic salmon (Tipsmark et al., 2008a, 2008b). In medaka, only *cldn28b* responded to salinity. This was seen during the initial 1-3 days of salinity acclimation, where a decreased expression in SW short-term mirrored an increase after FW transfer. However, no difference was detected between salinities in long-term acclimated-fish, suggesting a role of this paralog during initial gill remodeling. Thus, the regulatory expression patterns of gill Cldn4 related paralogs, as examined in the present study, may have species-specific functions possibly attributed to the ecology of the species (Tipsmark et al., 2008a, 2008b; Bagherie-Lachidan et al., 2009). The constitutive expression level of *cldn27a*, -28a and -30c in

medaka exposed to different salinity may suggest equal significance in FW and SW. The branchial epithelium of FW fish needs to be tight to limit the constant water entry and ions loss, while SW gills secrete sodium via “leaky” tight junction located between the ACs and MRCs. However, these SW ionocytes constitute only a minority of the epithelial cells (Perry and Walsh, 1989; Perry and Laurent, 1993). Even in SW, more than 80% of the epithelial surface is composed of PVCs, with deep TJs in between each other and when bordering to ACs and MRCs (Sardet et al., 1979; Evans et al., 2005). This suggests that SW gills are mainly a non-leaky epithelium which would support the need to limit water loss and ions intrusion. Our data suggests that *cldn27a*, *-28a* and *-30c* play a role in maintaining gill integrity and tightness between pavement cells and in the deeper cell layers of the filament epithelium. Consistent with this, *Cldn30c* are localized at epithelial cell-cell contacts on the zebrafish larval skin (Kwong and Perry, 2013) and between both PVCs and cells deeper in the branchial filament in Atlantic salmon (Engelund et al., 2012). In both species this isoform creates cation barrier as predicted by *in silico* analysis. Elevated expression of *cldn28b* during development of cultured-epithelium from rainbow trout suggests involvement in epithelium integrity (Sandbichler et al., 2011; Kolosov et al., 2014). The elevation of gill *Me-cldn28b* observed during the initial acclimation stage to FW could thus mirror important modifications of intercellular adhesion during the transition.

4.4 Claudins in medaka intestine

Medaka *cldn15a*, *-15b* and *-25* were found predominantly in the intestine, with some renal *cldn15a* expression (~10% of intestinal levels). This is in accordance with previous findings in Atlantic salmon (Tipsmark et al., 2010) and zebrafish (Bagnat et al., 2007). Similarly, studies on tissue distribution in *Fugu* found *cldn15a* in intestine and kidney and *cldn25* in the intestine, while *cldn15b* was below detection levels (Loh et al., 2004). In the medaka intestine, *cldn15b* responded to salinity change by a down-regulation in SW. In contrast, SW transfer of Atlantic salmon induced an up-regulation of both *cldn15a* and *-25b* (Tipsmark et al., 2010) and they were suggested to be involved in decreasing paracellular ionic permeability (Sundell et al., 2003; Tipsmark et al., 2010). This could, in theory, limit luminal salt loss from the lateral intercellular space and direct salt and water uptake to a transcellular route supported by elevated expression of aquaporins and Na⁺,K⁺-ATPase in salmonid enterocytes after SW entry (Seidelin et al., 2000;

Madsen et al., 2011). Given the leaky nature of the intestinal epithelium in general, this does not preclude a paracellular route of solute and water uptake in marine salmonids (Sundell and Sundh, 2012). In addition, zebrafish Cldn15 functions in paracellular cation-selective transport and is critical during intestinal lumen formation (Bagnat et al., 2007). In mammalian intestine, expression of Cldn2 and Cldn15 appears associated with formation of paracellular water and cation-selective pores involved in high paracellular transport-rates (Rosenthal et al., 2010; Günzel and Yu, 2013). The intestine of FW teleosts is involved in dietary ion uptake, while extensive uptake of NaCl in SW is thought to drive compensatory water uptake (Marshall and Grosell, 2006; Madsen et al., 2014). In medaka acclimated to FW, the presence of intestinal *cldn15a* and -25 may have an important role in hyper-osmoregulation. Furthermore, *cldn15b* with 10-fold higher expression in FW could be involved in paracellular cation (likely K⁺) transport. In rainbow trout, net transport of sodium in FW intestine is negligible, though there is a 80-90% uptake of K⁺ and Cl⁻ (Bucking and Wood, 2006). In SW, a decrease in transcript level of *Me-cldn15b* paralog was observed. We previously showed that aquaporins located in the apical membrane have a lower expression in SW than FW (Madsen et al., 2014). This suggests that intestinal water absorption in SW depends on paracellular water flux to a higher degree than in salmonids, and could possibly be associated with the change in *Me-cldn* paralog composition observed in the present study.

4.5 Claudins in medaka kidney

In the present study, two *cldn10b* splice variants were found to be expressed mainly in the kidney, similar to Cldn10 splice variants in mammals (Angelow et al., 2008). Our study is in accordance with studies in *Fugu* and zebrafish showing that *cldn10b* was restricted to the kidney (Loh et al., 2004; Baltzegar et al., 2013). In FW teleosts, the distal tubule is largely water impermeable and instrumental in producing hypotonic urine to compensate ions losses. Water elimination and salt retention occur via apical Na⁺,Cl⁻ cotransporter and Na⁺,K⁺,2Cl⁻ cotransporter type 2, driven by basolateral Na⁺,K⁺-ATPase (Engelund and Madsen, 2011; Kato et al., 2011). In marine fishes, the primary urine is supposedly produced by glomerular filtration in combination with the secretion of NaCl and water into the proximal nephron segments, linked to the active secretion of Mg²⁺ and SO₄²⁻ (Cliff and Beyenbach, 1992; Beyenbach, 2004). In the mammalian proximal tubule, the coordinated action of anion-selective and cation-selective Cldn

paralogs expressed in separate segments are thought to be involved in re-uptake of filtered chloride and sodium, respectively (Hou et al., 2013). While a number of studies have documented expression of a series of *cldn* paralogs in the teleost kidney (Loh et al., 2004; Baltzegar et al., 2013), the current study is to our knowledge the first to report a renal *cldn* responding to salinity. As discussed in the *in silico* analysis, medaka splice variant Cldn10b1 shares high similarity to mammalian cation-pore forming Cldn10b. Meanwhile, medaka splice variant Cldn10b2 is more similar to mammalian anion-pore forming Cldn10a (Van Itallie et al., 2006; Angelow et al., 2008). It is possible that elevated expression of the two medaka *cldn10b* in SW is important to paracellular NaCl re-absorption coupled to transcellular divalent ion secretion in the proximal tubule (Beyenbach, 2004), with each splice variant having selective ion selectivity.

4.5 Conclusion and perspectives

The present study of claudin proteins in osmoregulatory organs of the euryhaline medaka represents the first contribution to a new area of research in this very tractable model for understanding osmoregulatory physiology. We showed that putative barrier-forming claudins (*cldn27a*, *-28a*, *-28b* and *-30c*) are constitutively expressed in the gill of long-term acclimated FW and SW medaka. A series of *cldn10* paralogs (*cldn10c*, *-10d*, *-10e* and *-10f*) had high gill expression especially in SW suggesting a role in SW ionocyte function, including paracellular sodium extrusion. Intestinal and renal claudins with potential pore-forming capacity are regulated after long-term salinity change and thus probably involved the function of these osmoregulatory epithelia. In future studies, it will be imperative to analyze protein abundance and localization and to classify functional barrier and transport characteristics of the individual claudin proteins.

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15 17359.

1 **Table 1:** Quantitative PCR primers used to detect *cldn* transcript levels in Japanese medaka
2 (*Oryzias latipes*).
3

Target name	NCBI Acc. No.	Forward primer	Reverse primer
<i>Me-cldn10b1</i>	XM_004081778.1	gagatcgtggcttctgtgt	tcattggacgacactttcca
<i>Me-cldn10b2</i>	XM_004081779.1	cgccattattggtgctacag	ggcatttcagcacagtttt
<i>Me-cldn10c</i>	XM_004081941.1	cggatcaattttggtcttgg	gcgagattcctcctacaag
<i>Me-cldn10d</i>	XM_004066669.1	ccaccgccaactactactcc	atgcaggaacaccggtagag
<i>Me-cldn10e</i>	XM_004066670.1	caaagtggcctgttactggt	tggatgtgaggtccacaga
<i>Me-cldn10f</i>	XM_004081942.1	atcaaggtggcctgttactg	gagaaccgcgtaatctctgc
<i>Me-cldn15a</i>	XM_004079825.1	tgtcctcaaaggaggattg	tgatgtgaaggcgtaggag
<i>Me-cldn15b</i>	XM_004076466.1	caacatcacgcagcagttct	aaacctccacagatggcaag
<i>Me-cldn25</i>	XM_004078759.1	caagaaggcgcgatactttg	aggctctggatgactctgga
<i>Me-cldn27a</i>	XM_004075237.1	catgtgcatcatctccatcc	gctttgacttctcgtggtc
<i>Me-cldn28a</i>	XM_004075235.1	accgaaagcccagagtatcc	ggccgctattgtaggacttg
<i>Me-cldn28b</i>	XM_004076181.1	ggcacagccttcattggat	tgcactcgcctgtactctg
<i>Me-cldn30c</i>	XM_004076176.1	cagccctctctgagtaacg	ctccaatcagcatcagagca

- 1 **Table 2:** Amino acid sequences from house mouse (*Mus musculus*) and tiger pufferfish
- 2 (*Takifugu rubripes*), used to make the phylogenetic tree and the alignment analysis.

Species	Name	Gene Bank accession number
<i>Mus musculus</i>	Cldn3	NM_009902.4
	Cldn4	NM_009903.2
	Cldn10a	NM_023878.3
	Cldn10b	NM_021386.4
	Cldn15	BC023428.1
	ClarinI	BC145202.1
<i>Takifugu rubripes</i>	Cldn10a	AY554359.1
	Cldn10b	AY554396.1
	Cldn10c	AY554395.1
	Cldn10d	AY554391.1
	Cldn10e	AY554392.1
	Cldn15a	AY554365.1
	Cldn15b	AY554357.1
	Cldn25	AY554364.1
	Cldn26	AY554363.1
	Cldn27a	AY554381.1
	Cldn27b	AY554382.1
	Cldn27c	AY554370.1
	Cldn27d	AY554374.1
	Cldn28a	AY554383.1
	Cldn28b	AY554375.1
	Cldn28c	AY554373.1
	Cldn29a	AY554372.1
	Cldn29b	AY554371.1
	Cldn30a	AY554380.1
	Cldn30b	AY554384.1
	Cldn30c	AY554369.1
	Cldn30d	AY554376.1
	Cldn31	AY554351.1

3

4

Table 3: Summary of significant effects of salinity on claudin transcript levels in gill, intestine, and kidney. For short-term transfer experiments, upward and downward arrows indicate up- or down-regulation of transcript levels and horizontal arrows indicate no significant effect. The putative function indicated is based on *in silico* analysis as seen in Fig. 2.

	Paralog	Long-term acclimation	Short-term FW to SW	Short-term SW to FW	Putative function
Gill	<i>cldn-10c</i>	FW < SW	↗	↘	Cation pore-forming?
	<i>cldn-10d</i>	FW < SW	↗	↘	Cation pore-forming
	<i>cldn-10e</i>	FW < SW	↗	↘	Cation pore-forming
	<i>cldn-10f</i>	FW < SW	↗	↘	Cation pore-forming
	<i>cldn-27a</i>	FW ~ SW	→	→	Barrier
	<i>cldn-28a</i>	FW ~ SW	→	→	Barrier
	<i>cldn-28b</i>	FW ~ SW	↘	↗	Barrier
	<i>cldn-30c</i>	FW ~ SW	→	→	Barrier
Intestine	<i>cldn-15a</i>	FW ~ SW			Cation pore-forming
	<i>cldn-15b</i>	FW > SW			Cation pore-forming
	<i>cldn-25</i>	FW ~ SW			Cation pore-forming?
Kidney	<i>cldn-10b1</i>	FW < SW			Cation pore-forming
	<i>cldn-10b2</i>	FW < SW			Anion pore-forming

Legends

Figure 1. Consensus phylogenetic tree of claudins in the Japanese medaka. The tree was generated using claudin aa sequences from medaka (Me, green), *Fugu* (Fu, blue) and mouse (Mo, yellow).

Claudin genes classes used in Loh et al. (2004) were mentioned, based on the origin and phylogenetic relationships. The bootstrap consensus tree was inferred from 1000 replicates and values > 70% are given.

Figure 2. Alignment of the 1st extracellular loop (ECL1) of claudins in the Japanese medaka (Me, grey) and mouse (Mo).

Aa residues are highlighted in red when acidic, in blue when basic. The bracket indicates area after the 2nd Cys residues particularly involved into selective permeability. Residues yellow highlighted indicate position essential for ion selectivity (for more information, see: Angelow et al., 2008; Li et al., 2013) *: identical residue; :: conserved residue; .: semi-conserved residue.

Figure 3. Claudins transcript abundance in various organs from Japanese medaka in FW (*cldn10b1*, A; *cldn10b2*, B; *cldn10c*, C; *cldn10d*, D; *cldn10e*, E; *cldn10f*, F; *cldn15a*, G; *cldn15b*, H; *cldn25*, I; *cldn27a*, J; *cldn28a*, K; *cldn28b*, L; *cldn30c*, M).

The expression of each claudins in kidney, intestine, muscle and liver are expressed in % of the gill abundance. Bars represent the mean value $\pm$ s.e.m. ($N = 4$). Expression levels were normalized against the geometric mean of three normalization genes. Shared letters above the bars indicate not significantly difference in expression between organs (n.d. indicates no detection).

Figure 4. Claudins transcript abundance in the osmoregulatory organs of Japanese medaka long-term acclimated to either FW or SW (*cldn10b1*, A; *cldn10b2*, B; *cldn10c*, C; *cldn10d*, D; *cldn10e*, E; *cldn10f*, F; *cldn15a*, G; *cldn15b*, H; *cldn25*, I; *cldn27a*, J; *cldn28a*, K; *cldn28b*, L; *cldn30c*, M).

The expression of each claudin is expressed in % of FW gill abundance. Bars represent the mean value $\pm$ s.e.m. ($N = 6$). of fish acclimated to either FW or SW. Expression levels were normalized against the geometric mean of three normalization genes. Asterisks above the bars

1 indicate significant differences compared to the FW-gill expression: **: $p < 0.01$ and *: $p < 0.05$
2 (n.d. indicates no detection). FW: fresh water; SW: seawater.

3 **Figure 5. Response in transcript abundance of prevailing branchial claudin paralogs, to**
4 **FW-to-SW transfer at short-term in Japanese medaka (*cldn10c*, A; *cldn10d*, B; *cldn10e*, C;**
5 ***cldn10f*, D; *cldn27a*, E; *cldn28a*, F; *cldn28b*, G; *cldn30c*, H).**

6 Fish were transferred from FW to SW or FW to FW as a control and gills were sampled after 1
7 and 3 days ($N = 10$). The expression of each claudin is expressed in % of FW gill abundance.
8 Bars represent the mean value + s.e.m. Expression levels were normalized against the geometric
9 mean of three normalization genes. Asterisks next to SW indicate significant overall effect of
10 salinity: ***: $p < 0.001$, **: $p < 0.01$. FW: fresh water; SW: seawater.

11 **Figure 6. Response in transcript abundance of prevailing branchial claudin paralogs, to**
12 **SW-to-FW transfer at short-term in Japanese medaka.**

13 Fish were transferred from SW to FW or SW to SW as a control and gills were sampled after 1
14 and 3 days ($N = 10$). The expression of each claudin is expressed in % of SW gill abundance.
15 Bars represent the mean value + s.e.m. Expression levels were normalized against the geometric
16 mean of three normalization genes. Asterisks next to FW indicate significant overall effect of
17 salinity: ***: $p < 0.001$, **: $p < 0.01$ and *: $p < 0.05$. FW: fresh water; SW: seawater.

18

1 **Figure 1**

1 **Figure 2**

2

1 Figure 3

1 **Figure 4**

1 **Figure 5**

1 **Figure 6**